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Inhibition of varicella–zoster virus *in vitro* by human peripheral blood mononuclear cells

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SUMMARY

A functional *in vitro* assay of cell-mediated immunity to varicella–zoster virus (VZV) is described. This procedure uses an enzyme-linked immunosorbent assay (ELISA) to measure the inhibitory effect of human peripheral blood mononuclear cells on VZV antigen production by VZV-infected cell monolayers. When mononuclear cells from VZV-immune, tetanus-immune donors were stimulated with either VZV antigen or tetanus toxoid they reduced VZV antigen production. In contrast, mononuclear cells from VZV-nonimmune, tetanus-immune donors reduced VZV antigen only when stimulated with tetanus toxoid, but not when stimulated with VZV antigen. Cell-free supernatants recovered from the VZV inhibition assays contained the anti-VZV activity. The magnitude of the anti-VZV activity of the supernatants equalled the inhibition observed when the stimulated mononuclear cells were added to the VZV-infected monolayers. Treatment of either mononuclear cells or supernatants with anti-interferon gamma antibody indicated that their VZV inhibitory capability was largely due to the production of interferon gamma by stimulated mononuclear cells.

Keywords varicella–zoster virus gamma interferon

INTRODUCTION

Varicella–zoster virus (VZV) infections pose a serious threat to individuals with impaired cell-mediated immunity (CMI), but not to those with impaired humoral immunity, thus indicating that CMI is essential for recovery from VZV infections (Merigan & Stevens, 1971; Arvin *et al.*, 1986). The VZV-specific CMI responses of peripheral blood mononuclear cells (PBMC) from normal immune individuals have been demonstrated in a variety of ways, including proliferative response to VZV antigens (Jordan & Merigan, 1974; Zaia, Leary & Levin, 1978), antibody-dependent cellular cytotoxicity (Kamiya *et al.*, 1982; Gershon & Steinberg 1981), interferon production (Armstrong & Merigan, 1971), natural killer cell activity (Tilden *et al.*, 1986), and cytotoxic T cell activity (Hayward *et al.*, 1986). A direct functional assay, namely the ability of PBMC to inhibit the growth of VZV in tissue culture has also been described (Gershon, Steinberg & Smith, 1976; Gershon & Steinberg, 1979a; 1979b; 1981).

In the assay described below we utilized an enzyme-linked immunosorbent assay (ELISA) to measure the ability of PBMC to inhibit the growth of VZV in cultured fibroblasts. The role of PBMC subsets and lymphokines in the observed viral inhibition was analysed.

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MATERIALS AND METHODS

Target cells

Human embryonic lung fibroblasts (HELF) obtained from J. Waner (University of Oklahoma, Norman, OK) were used at passages 17 to 25. Human bone-marrow fibroblasts were obtained and stored by a method described previously (Bowden *et al.*, 1984).

Virus

VZV vaccine strain (Oka) (lot 867; CH198; Merck, Sharp & Dohme Research Laboratories, West Point, PA) and our laboratory strain (Cp 5, 262; Passage 14) were used. Cell-free VZV was prepared by sonication of infected cells, and stored at a titre of $1{\cdot}2 \times 10^5$ pfu/ml in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (DUL-10) and 7% sorbitol at $-70°C$. The VZV titre was determined by standard plaque titration in HELF using 12-well plastic plates (Linbro; Flow Labs, Inc., McLean Virginia) after fixation with methanol-acetic acid and staining with crystal violet.

Mononuclear cell donors

Eight young adults with a history of varicella in the distant past (Group A), eight young adults with varicella in the previous 6 months (Group B), and seven VZV-susceptible adults (Group C) were PBMC donors. All donors were otherwise healthy.

Anti-VZV antibody determination
Sera from donors was tested by the fluorescent anti-membrane antibody test (Zaia & Oxman, 1977).

Preparation and stimulation of PBMC
Heparinized or defibrinated blood was layered on Ficoll-Paque (Pharmacia, Piscataway, NJ) and centrifuged at 400 *g* for 30 min at 20°C. The PBMC at the interface were washed twice with Hanks' balanced salt solution (HBSS) (400 *g* for 10 min at 4°C) and once with RPMI-1640. PBMC were stimulated at 10^6 cells/ml in RPMI-1640 containing L-glutamine (0·3 mg/ml, Gibco, Grand Island, NY), penicillin (200 units/ml), kanamycin (100 μg/ml), 10% autologous serum, and the appropriate antigen. The VZV antigen was prepared from a glycine-buffer (pH 9·0) extract of VZV-infected HELF (Zaia *et al.*, 1978). Control antigen was prepared from uninfected HELF. These antigens were used at a final dilution of 1/100. Tetanus toxoid (TT) (gift of Wyeth Laboratories, Dallas, TX), dialysed against HBSS, was used at a final concentration of 2 flocculation units/ml. PBMC were incubated with antigen at 37°C in a 5% CO_2 atmosphere for 6 days. The cells were then resuspended, an aliquot removed for studies of ^{3}H-thymidine uptake, and the remainder separated on Ficoll-Paque, washed, and tested for anti-viral activity.

^{3}H-Thymidine uptake assay
Stimulated PBMC at 10^6 cells/ml in RPMI-1640 were distributed in 0·2 ml aliquots, in triplicate, into microtitre wells (Linbro. No. 76-013-05), and 0·25 μCi of ^{3}H-thymidine (20 Ci/mmol) in 50 μl of RPMI-1640 was added to each well. After 6 h at 37°C in 5% CO_2, the wells were harvested onto glass fibre discs and counted in a scintillation counter. The stimulation index (SI) was calculated as the mean counts for cells stimulated with VZV or TT divided by the mean count for cells stimulated with control antigen.

Anti-viral assay
Infection of monolayers. The wells of 96-well flat-bottomed microtitre plates (Falcon, minitest III, Oxnard, CA) were seeded with $3·5 \times 10^4$ HELF in 0·2 ml Dul-10. The edge wells were filled with sterile water only. When the monlayers were confluent, the medium was aspirated and VZV in 0·1 ml of Dulbecco's modified Eagle's medium containing 2% fetal calf serum and antibiotics (Dul-2) was added. The inoculum was 50 pfu/well for the laboratory VZV strain and 100 pfu/well for the vaccine strain, corresponding to a multiplicity of infection of $0·63 \times 10^{-3}$ and $1·25 \times 10^{-3}$, respectively.

Antigen reduction by MNC. After 4 h adsorption at 37°C in 5% CO_2 the wells were washed, 1×10^5 PBMC in 0·2 ml of Dul-2 were added, and the plates incubated at 37°C in 5% CO_2. Controls consisted of uninfected and infected wells fed with Dul-2 only. After 3 days the medium was removed and the ELISA performed on the monolayers. A minimum of three replicate wells was used for each experimental condition.

Antigen reduction by supernatants. After the PBMC had been incubated with the infected monolayers for 3 days, the medium was removed, centrifuged at 400 *g* for 10 min and the supernatants stored at −70°C. The antigen reduction assay was performed by adding 0·2 ml of cell-free supernatants onto VZV-infected HELF monolayers. The ELISA was performed 3 days later. For experiments evaluating the interferon-like activity of supernatants we added supernatants to uninfected monolayers for 24 h, and removed them prior to VZV infection.

ELISA for quantification of VZV antigen. This method has been described in detail elsewhere (Berkowitz & Levin, 1985). Briefly, the monolayers were washed and incubated sequentially with varicella-zoster immune globulin (VZIG, Massachusetts Public Health Biologic Laboratories), peroxidase-conjugated goat anti-human immunoglobulin G (Tago, Inc., Burlingame, CA) and the substrate. The absorbance at 405 nm of all wells was determined when the mean A_{405} of the virus control wells was 1·0 to 1·5.

Treatment of PBMC and supernatants with anti-interferon antibodies
Stimulated PBMC and supernatants were treated with rabbit antiserum to human interferon gamma (NIH research reagent, No. G-034-501-565, Bethesda, Maryland) for 1 h at 37°C prior to testing for anti-viral activity. The amount of antiserum used (26 μl) was sufficient to neutralize 50 units of interferon gamma per 5×10^5 stimulated PBMC or per millilitre of supernatant. Normal rabbit serum controls were performed using NIH research reagent, cat no. 035501565. In addition supernatants were treated similarly with sheep antiserum to human leucocyte interferon alpha (NIH research reagent, cat. no. G-026-502-568) and control sheep antiserum (NIH research reagent, no. G-027-501-568) in an amount (15 μl) sufficient to neutralize 12,000 units of alpha interferon per millilitre of supernatant.

Depletion of adherent cells
Two millilitres of stimulated PBMC, at 1 to $2·5 \times 10^6$ cells/ml in RPMI-1640 with L-glutamine and 10% autologous serum, was placed in a 60×15 mm plastic Petri dish (Falcon, no. 3002, Oxnard, CA) for 2 h at 37°C in 5% CO_2. Non-adherent cells were then removed and plated on a fresh Petri dish. The non-adherent cells were then washed in Dul-2 and a non-specific esterase stain, using alpha-naphthyl acetate as the substrate, was used to enumerate residual esterase-positive cells.

Fluorescence-activated cell sorting
Mononuclear cells stimulated for 6 days with VZV and control antigen were stained with CD4 or CD8 antibodies (Ortho Diagnostics, Raritan, NJ) conjugated to FITC according to the maker's instructions. They were washed twice and resuspended in HBSS for sorting on an EPICS C gated on forward angle light scatter and log green fluorescence. The anti-viral assay was performed 3 days after adding 5×10^4 CD4-positive or CD8-positive cells in 0·2 ml Dul-2 to triplicate microtitre wells containing VZV-infected fibroblasts.

RESULTS

Effect of unstimulated PBMC from VZV-immune donors on VZV infection in HELF monolayers
Unstimulated MNC from five donors reduced VZV antigen production by the VZV infected monolayers only $6 \pm 8\%$ (data not shown). When PBMC were added to the infected monolayers, together with soluble VZV antigen, as reported by others (Gershon *et al.*, 1976), the reduction was still only $3 \pm 5\%$.

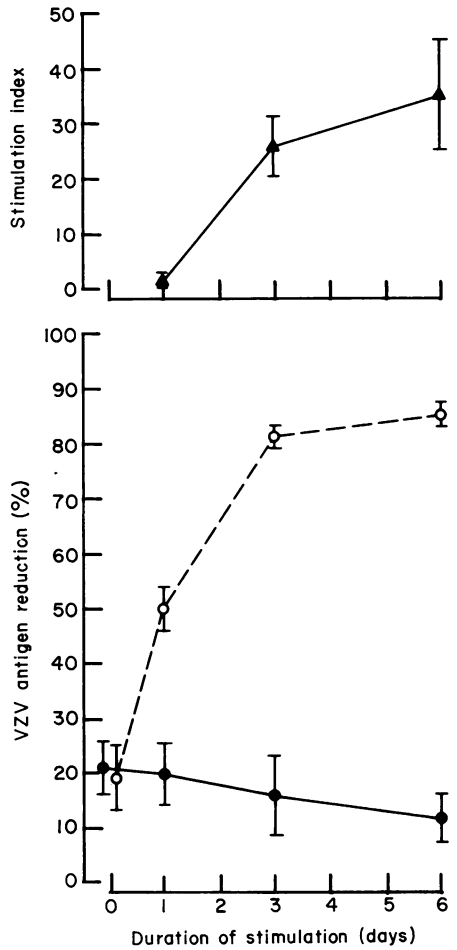

Fig. 1. Time course of the effect of stimulation with VZV antigen on the stimulation index of PBMC and the ability of PBMC to limit VZV infection. Stimulation Index was determined after stimulation with VZV antigen (▲). The percentage VZV antigen reduction was measured in a 3-day assay after stimulation with VZV antigen (○) or control antigen (●). Brackets indicate ±2 standard errors of the mean. Data from single PBMC donor.

Time course of PBMC stimulation by VZV antigen

In contrast to the results with unstimulated PBMC, cells stimulated with VZV antigen added to VZV-infected monolayers reduced VZV antigen production by 80–90% (Fig. 1). The percentage reduction in VZV antigen production correlated positively with the time that PBMC were cultured with VZV antigen ($P = 0{\cdot}23$ Day 0; $P < 0{\cdot}05$ days 1, 3 and 6 using two-tailed t-test) and with the PBMC proliferative response ($r = 0{\cdot}86$, $r^2 = 0{\cdot}74$). Both were maximal after 5–6 days of stimulation.

Lymphoproliferative responses of stimulated PBMC

The stimulation index of PBMC from donor groups A, B, and C after stimulation with VZV or TT antigens is shown in Fig. 2.

Effect of stimulated PBMC on VZV infection in HELF

The anti-VZV effect of PBMC from donor groups A, B, and C stimulated for 6 days with VZV, control, or TT antigens is summarized in Fig. 3. Both VZV- and TT-stimulated PBMC

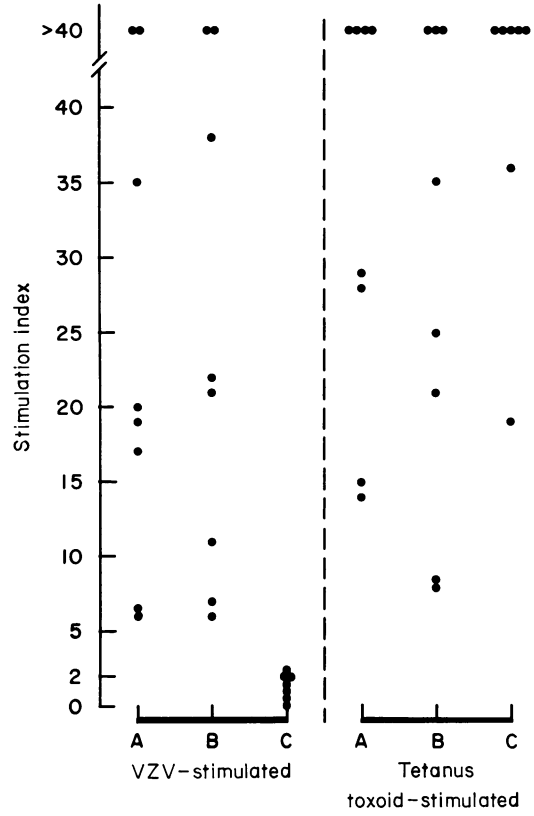

Fig. 2. Stimulation index as measured by ^{3}H-thymidine uptake for VZV- and TT-stimulated PBMC from three patient groups.

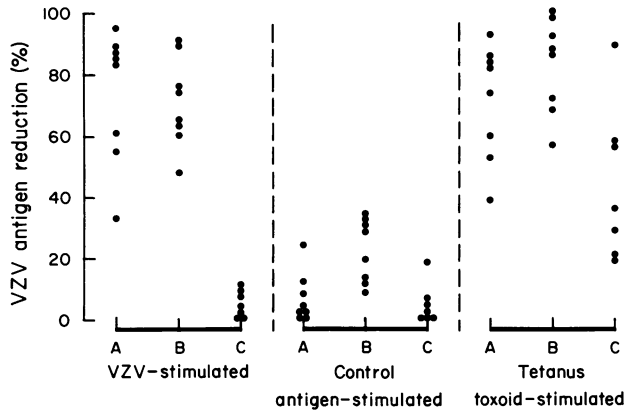

Fig. 3. Percentage VZV antigen reduction by PBMC from three patient groups as determined by ELISA.

from VZV-immune, tetanus-immune donors reduced VZV antigen production. However, PBMC from VZV-non-immune, tetanus-immune donors reduced VZV antigen production only when stimulated with TT antigen and not with VZV antigen.

Effect on the anti-viral activity of PBMC of haplotype sharing between the donor PBMC and the VZV-infected fibroblasts

A bone-marrow fibroblast line matched at two HLA loci with one PBMC donor and at no loci with a second PBMC donor was infected with VZV. Both donors were VZV-immune. Stimulated PBMC from both donors had a strong anti-viral effect (Table 1).

Table 1. Effect of unstimulated and stimulated PBMC from VZV-immune donors on VZV-infection in bone marrow fibroblast monolayers with and without shared HLA A and B antigens

PBMC donor	HLA antigens shared with Target-cell donor*	Reduction in VZV antigen (%) by PBMC stimulated with† Control	VZV	TT
1	2	13	44	57
2	none	13	84	79

* Haplotype for bone-marrow fibroblast donor was A_1, A_3, B_7, B_{27}; for donor 1 was A_2 A_3 B_7, B_{12}; for donor 2 was A_2, A_2, B_5, B_{16}.

† Percentage reduction as defined in Materials and Methods. PBMC were exposed to antigen for 6 days prior to performing the VZV antigen reduction assay.

Table 2. Effect of adherent cell depletion on the anti-VZV activity of stimulated PBMC*

PBMC Donor	Reduction in VZV antigen (%)† Untreated	Adherent cell-depleted
1	74	28
2	72	−3
3	86	40
Mean ± s.d.	77·3 ± 7·6‡	21·0 ± 22·2

* PBMC are cultured for 6 days with VZV antigen prior to depletion of adherent cells.

† Percentage reduction is as defined in Materials and Methods.

‡ Difference between untreated and depleted PBMC is significant ($P = 0{\cdot}03$). Student's paired *t*-test.

Effect of depletion of adherent cells on the anti-viral activity of stimulated PBMC

PBMC from three VZV-immune donors were stimulated with VZV antigen for 6 days and then depleted of 99% of plastic adherent cells. Removal of these cells from stimulated PBMC markedly diminished the VZV-antigen reduction by PBMC (Table 2).

Effect of supernatants from the VZV-antigen reduction assay on VZV infection

Cell-free supernatants recovered from 3-day VZV-antigen reduction assays had anti-VZV activity when subsequently tested directly on VZV-infected monolayers. This inhibition closely correlated with that produced by the stimulated PBMC from which the supernatant was obtained (Fig. 4; $r = 0{\cdot}75$; $P < 0{\cdot}001$).

Role of interferon gamma

Evidence that the anti-VZV effect was due to interferon gamma came from two sources. Firstly an anti-viral state was induced in uninfected HELF monolayers by incubation with supernatants for 24 h. The supernatant was removed prior to infecting with VZV. Performance of the ELISA 3 days later revealed 98% VZV antigen reduction in wells pretreated with supernatants from VZV-stimulated PBMC and 38% VZV reduction in wells pretreated with supernatants from control antigen-stimulated PBMC. Secondly when supernatants generated by the interaction of VZV-stimulated PBMC with VZV-infected HELF were treated with rabbit anti-interferon gamma antibody the anti-VZV activity was reduced from 92% to 2%. Treatment of these supernatants with sheep anti-interferon alpha antibody did not reduce the anti-VZV activity. These are the mean values of duplicate experiments using PBMC from a single donor. No anti-VZV antibody was detected in these supernatants using the fluorescent anti-membrane antibody test.

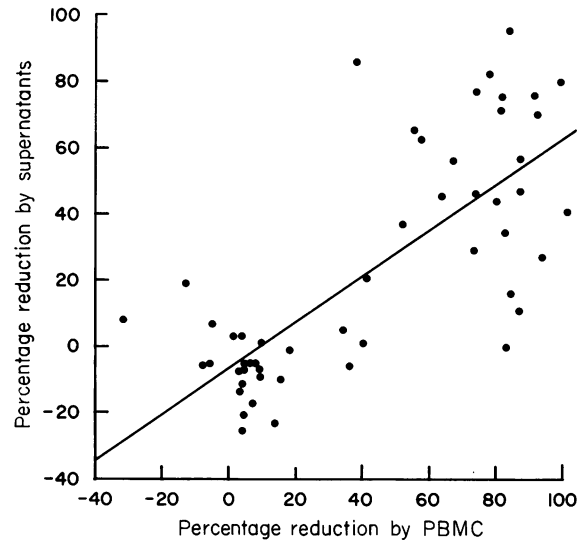

Fig. 4. Relationship between VZV-antigen reduction by supernatants and by stimulated PBMC.

Table 3. Effect of anti-interferon gamma antibody on the ability of stimulated PBMC to reduce VZV antigen production in VZV-infected HELF monolayers

Treatment	Reduction in VZV antigen (%) by PBMC* stimulated with Control	VZV	TT
None	23	90	99
Anti-interferon gamma	0	0	5

* PBMC from one donor stimulated for 6 days.

Effect of treatment of stimulated PBMC with anti-interferon gamma antibody

After the 6-day stimulation with VZV, control, or TT antigens, PBMC were placed onto VZV-infected HELF monolayers in the presence of rabbit anti-interferon gamma and the ELISA performed 3 days later. Complete inhibition of the anti-viral activity of these PBMC was noted (Table 3).

Effect of lymphocyte subsets on VZV antigen reduction
Experiments were undertaken to determine which subsets of lymphocytes could limit production of VZV antigen. Populations of CD4 and CD8 cells obtained from VZV antigen-stimulated PBMC from a VZV-immune donor each reduced VZV antigen by 93%. CD4 and CD8 cells separated from control antigen-stimulated PBMC from the same donor reduced VZV antigen 28% and 45% respectively.

DISCUSSION

Our data indicate that stimulated blood mononuclear cells suppress the production of VZV antigen in monolayers of permissive cells over a 3-day culture period. A reduction in the amount of VZV replication in the infected monolayer is the most likely mechanism, as we have shown previously that the amount of VZV antigen detected by the ELISA method correlates with the amount of VZV replication in the target cells (Berkowitz *et al.*, 1985).

Freshly isolated PBMC did not reduce VZV antigen production. A necessary condition for this suppression was the activation of T cells. VZV antigen was a sufficient stimulus for donors immune to VZV, and tetanus toxoid was sufficient for donors immune to this antigen. The lack of antigen specificity for the induction of VZV suppression suggested that direct recognition of VZV antigen on the target cell surface was unnecessary. This view was supported by the lack of requirement for HLA matching between the effector PBMC and the target monolayer. This result is consistent with the lack of HLA restriction previously observed in a herpes simplex virus plaque reduction assay (Leibson, Hunter-Laszlo & Hayward, 1986).

The lack of antigen specificity or HLA matching between the mononuclear cells and the fibroblast monolayer suggested that lymphokines might mediate the VZV suppression phenomenon. The anti-viral effect induced by culture supernatants in target cells in the absence of cells or antibody suggested that interferon was present. Inhibition of the effect by type-specific anti-interferon antibodies established a role for interferon gamma. When the stimulated PBMC were sorted into CD4 and CD8 subsets, both populations mediated the VZV suppression. This data is compatible with previous reports that both T cell subsets are capable of producing gamma interferon following stimulation (Kasahara *et al.*, 1983). The finding that removal of monocytes ($< 1\%$ remained) interfered with the VZV suppression phenomenon is not surprising, as continuing activation of VZV-specific T cells is known to require accessory cells (Pontesilli *et al.*, 1987). Why monocyte removal resulted in such interference, given that CD4 and CD8 subsets were active alone, may be explained by the fact that plastic adherence removes both monocytes and any cells which may adhere to them, like antigen-specific T cells. Cells sorted by fluorescence-activated cell sorting had no opportunity for adherence to monocytes as they were resuspended frequently, hence activated cells would have been present in equal numbers.

The *in vitro* VZV suppression test was originally proposed using freshly isolated PBMC as a correlate of cell-mediated immunity (Gershon *et al.*, 1976; Gershon & Steinberg 1979a; 1981). Our study failed to reproduce these data although stimulated PBMC were clearly active. This result is consistent with our previous observations on the paucity in the peripheral blood of lymphocytes which respond to VZV antigen. In VZV-immune individuals this responder cell frequency is 1 in 5000 to 1 in 50 000, depending on age (Hayward & Herberger, 1987). The inhibition of VZV plaque formation reported by Gerhson *et al.* (1976) in cultures containing only 500 to 1000 PBMC was therefore probably mediated by mechanisms other than VZV-specific T cells.

The inhibition of VZV antigen in our assay appears to depend on interferon gamma production resulting from T cell activation. This conclusion also differs from that of Gershon *et al.*, who suggested that a direct cell-mediated cytotoxic mechanism was responsible for inhibiting virus growth because they were unable to demonstrate the presence of interferon (Gershon & Steinberg, 1979a). At the same time they excluded inhibition by interferon (Gershon & Steinberg, 1979a). Nevertheless interferon gamma clearly was present in our cultures and appeared sufficient to account for the anti-viral activity of activated T cells. Our finding of interferon gamma activity is probably a function of the large number of PBMC which we added to VZV-infected monolayers, and their prior activation with VZV antigen.

To the several VZV-specific cell-mediated immune responses which have been described *in vitro* we add the production of interferon gamma by CD4 and CD8 lymphocytes. The interaction and relative contributions of these responses *in vivo* is unclear. However, interferon gamma production is likely to be a useful measure of immune function. Since interferon gamma production is directly measurable after in-vitro stimulation of mononuclear cells this would appear to be a more direct approach to assessing immune potential than the relatively cumbersome virus suppression assay described here.

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